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(54) Title: MULTIANALYTE MOLECULAR ANALYSIS

(57) Abstract: The present invention provides methods and apparatus for the application of a particle array in bioassay format to perform qualitative and/or quantitative molecular interaction analysis between two classes of molecules (an analyte and a binding agent). The methods and apparatus disclosed herein permit the determination of the presence or absence of association, the strength of association, and/or the rate of association and dissociation governing the binding interactions between the binding agents and the analyte molecules. The present invention is especially useful for performing multiplexed (parallel) assays for qualitative and/or quantitative analysis of binding interactions of a number of analyte molecules in a sample.

MULTIANALYTE MOLECULAR ANALYSIS

FIELD OF THE INVENTION

The present invention relates to mutiplexed bioassays for analyzing binding interactions between analytes and binding agents, including methods for determining the affinity constants and kinetic properties associated with analyte-binding agent interactions.

BACKGROUND OF THE INVENTION

The imprinting of multiple binding agents such as antibodies and oligonucleotides on planar substrates in the form of spots or stripes facilitates the simultaneous monitoring of multiple analytes such as antigens and DNA in parallel ("multiplexed") binding assays. The miniaturization of this array format for increasing assay throughput and studying binding kinetics are described, for example, in R. Ekins, F. W. Chu, *Clin. Chem.* **37**, 955-967 (1991); E. M. Southern, U. Maskos, J. K. Elder, *Genomics* **13**, 1008-1017 (1992). In recent years, this approach has attracted substantial interest particularly in connection with performing extensive genetic analysis, as illustrated in G. Ramsay, *Nat. Biotechnol.* **16**, 40-44 (1998); P. Brown, D. Botstein, *Nat. Genet.* **21**, 33-37 (1999); D. Duggan, M. Bittner, Y. Chen, P. Meltzer, J. M. Trent, *Nat. Genet.* **21**, 10-14 (1999); R. Lipshutz, S. P. A. Fodor, T. R. Gingeras, D. J. Lockhart, *Nat. Genet.* **21**, 20-24 (1999).

The present invention provides methods and apparatus for the application of a particle array in bioassay format to perform qualitative and/or quantitative molecular interaction analysis between two classes of molecules (an analyte and a binding agent). The methods and apparatus disclosed herein permit the determination of the presence or absence of association, the strength of association, and/or the rate of association and dissociation governing the binding interactions between the binding agents and the analyte molecules. The present invention is especially useful for performing multiplexed (parallel) assays for qualitative and/or quantitative analysis of binding interactions.

The terms "analyte" and "binding agent" refer to molecules involved in binding interactions. In one example, analyte and binding agent include DNA or RNA fragments (e.g., oligonucleotide), and binding of those fragments to their complementary sequences (hybridization) is analyzed. In another example, binding interactions between ligands and receptors are analyzed. Examples of analytes and binding agents also include aptamers, peptides and proteins (e.g., antibodies), antigens, and small organic molecules.

The term "particles" refer to colloidal particles, including beaded polymer resins ("beads").

The present invention also provides automated, on-demand fabrication of planar arrays composed of a selected mixture of chemically distinct beads (e.g., encoded beads) which are disposed on a substrate surface in accordance with a selected spatial configuration, as described above. In this approach, the beads are functionized to display binding agents. For example, the binding agents may be attached to the beads, preferably by covalent bond. The subsequent quality control and performance evaluation are conducted off-line and are independent from the process of array assembly. The separation of steps such as bead encoding, functionalization and testing; substrate design, processing and evaluation; custom assembly of application-specific arrays; and on-line decoding of

Fig. 8 is an illustration of steps in the decomposition of assay images according to bead type by application of the image analysis algorithm summarized in Fig. 7.

Fig. 9 is an illustration of optically programmable array assembly of random encoded arrays

Fig. 10 is an illustration of an array composed of random encoded subarrays

Fig. 11 is an illustration of stations in an automated chip-scale bead array manufacturing and QC process

Fig. 12 is an illustration of quantitative binding curves for two cytokines

Fig. 13 is an illustration of array design for polymorphism analysis

Fig. 14 is a fluorescence micrograph of assay and decoding images recorded from one subarray shown in Fig. 13 in the course of polymorphism analysis

Fig. 15 is an illustration of assay results in the form of intensity histograms obtained from the analysis of assay images such as the one illustrated in Fig. 14.

Fig. 16 is an illustration of design of a "looped probe" for hybridization assays

Fig. 17A and 17B are fluorescence micrographs of assay and decoding images recorded in the course of the analysis of multiple cytokines

Fig. 18A and 18B are illustrations of numerical simulations of cross-correlations in receptor-ligand systems with multiple competing receptor-ligand interactions

Fig. 19 is an illustration of numerical simulations of receptor-ligand association and disassociation kinetics

Fig. 20 is an illustration of integrated sample capture using magnetic capture beads and array-based detection using READ

Fig. 21 is an illustration of multi-step assays using encoded magnetic beads to integrate gene-specific capture, on-bead reverse transcription and post-assay array assembly

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

samples. The integration of LEAPS with microfluidics produces a self-contained, miniaturized, optically programmable platform for parallel protein and DNA analysis. LEAPS refers to methods of moving particles suspended in the interface between an electrolyte solution and an electrode and is described in U.S. Patent Application Serial No. 09/171,550 (also PCT International Application No. PCT/US97/08159) entitled Light-controlled Electrokinetic Assembly of Particles near Surfaces, which is incorporated herein by reference in its entirety. Also incorporated herein by reference in its entirety is U.S. Patent Applications Serial No. 09/397,793 (also PCT International Application PCT/US00/25466), entitled "System and Method for Programmable Pattern Generation.

In certain embodiments of the present invention, chemical encoding may be accomplished by staining beads with sets of optically distinguishable tags, such as those containing one or more fluorophore dyes spectrally distinguishable by excitation wavelength, emission wavelength, excited-state lifetime or emission intensity. The optically distinguishable tags made be used to stain beads in specified ratios, as disclosed, for example, in Fulwyler, US 4,717,655 (Jan 5, 1988). Staining may also be accomplished by swelling of particles in accordance with methods known to those skilled in the art, [Molday, Dreyer, Rembaum & Yen, J. Mol Biol 64, 75-88 (1975); L. Bangs, "Uniform latex Particles, Seragen Diagnostics, 1984]. For example, up to twelve types of beads were encoded by swelling and bulk staining with two colors, each individually in four intensity levels, and mixed in four nominal molar ratios. Combinatorial color codes for exterior and interior surfaces is disclosed in International Application No. PCT/US 98/10719, which is incorporated herein by reference in its entirety.

Beads are functionalized by binding agent molecules attached thereto, the molecule including DNA (oligonucleotides) or RNA fragments, peptides or proteins, aptamers and small organic molecules in accordance processes known in the art, e.g., with one of several coupling reactions of the known art (G. T. Hermanson, *Bioconjugate Techniques* (Academic Press, 1996); L. Illum, P. D. E. Jones, *Methods in Enzymology* **112**, 67-84

beads are introduced into the gap. When an AC voltage is applied to the gap, the beads form a random encoded array on the second electrode (e.g., "chip"). And, also using LEAPS, an array of beads may be formed on a light-sensitive electrode ("chip"). Preferably, the sandwich configuration described above is also used with a planar light sensitive electrode and another planar electrode. Once again, the two electrodes are separated by the a gap and contain an electrolyte solution. The functionalized and encoded beads are introduced into the gap. Upon application of an AC voltage in combination with a light, the beads form an array on the light-sensitive electrode.

In certain embodiments, the application-specific bead arrays useful in the present invention may be produced by picking aliquots of designated encoded beads from individual reservoirs in accordance with the specified array composition and "pooled"; aliquots of pooled suspension are dispensed onto selected substrate (e.g., chips) in a manner preventing the initial fusion of aliquots. Aliquots form a multiplicity of planar random subarrays of encoded beads, each subarray representing beads drawn from a distinct pool and the physical array layout uniquely corresponding to the identity of aliquots drawn from pooled bead populations.

Planar arrays or assemblies of encoded on a substrate which are chemically or physically encoded may be used. To this, spatial encoding may also be added to increase the number of assays that may be conducted. Spatial encoding, for example, can be accomplished within a single fluid phase in the course of array assembly by invoking Light-controlled Electrokinetic Assembly of Particles near Surfaces (LEAPS) to assemble planar bead arrays in any desired configuration in response to alternating electric fields and/or in accordance with patterns of light projected onto the substrate. LEAPS creates lateral gradients in the impedance of the interface between silicon chip and solution to modulate the electrohydrodynamic forces that mediate array assembly. Electrical requirements are modest: low AC voltages of typically less than $10V_{pp}$ are applied across a fluid gap of typically 100 μm between two planar electrodes. This assembly process is rapid and it is optically programmable: arrays containing thousands of beads are formed within seconds under electric

In certain embodiments, the particle arrays may be immobilized by chemical means, e.g, by forming a composite gel-particle film. In one exemplary method for forming such gel-composite particle films, a suspension of microparticles is provided which also contain all ingredients for subsequent in-situ gel formation, namely monomer, crosslinker and initiator. The particles are assembled into a planar assembly on a substrate by application of LEAPS, e.g., AC voltages of 1-20 V_{p-p} in a frequency range from 100's of hertz to several kilohertz are applied between the electrodes across the fluid gap. Following array assembly, and in the presence of the applied AC voltage, polymerization of the fluid phase is triggered by thermally heating the cell ~ 40-45^{0}C using an IR lamp or photometrically using a mercury lamp source, to effectively entrap the particle array within a gel. Gels may be composed of a mixture of acrylamide and bisacrylamide of varying monomer concentrations from 20% to 5% (acrylamide : bisacrylamide = 37.5 : 1, molar ratio), or any other low viscosity water soluble monomer or monomer mixture may be used as well. Chemically immobilized functionalized microparticle arrays prepared by this process may be used for a variety of bioassays, e.g., ligand receptor binding assays.

In one example, thermal hydrogels are formed using azodiisobutyramidine dihydrochloride as a thermal initiator at a low concentration ensuring that the overall ionic strength of the polymerization mixture falls in the range of ~ 0.1mM to 1.0mM. The initiator used for the UV polymerization is Irgacure 2959® (2-Hydroxy-4'-hydroxyethoxy-2-methylpropiophenone, Ciba Geigy, Tarrytown, NY). The initiator is added to the monomer to give a 1.5 % by weight solution.

In certain embodiments, the particle arrays may be immobilized by mechanical means. For example, an array of microwells may be produced by standard semiconductor processing methods in the low impedance regions of the silicon substrate. The particle arrays may be formed using such structures by, e.g., utilizing LEAPS mediated hydrodynamic and ponderomotive forces are utilized to transport and accumulate particles on the hole arrays. The A.C. field is then switched off and particles are trapped into

characteristic associated with those beads. Preferably, chemically distinguishable characteristics include chemical molecules including flurophore dyes, chromophores and other chemical molecules that are used for purposes of detection in binding assays.

The detection of the chemically or physically distinguishable characteristic and the detecting of the optical signature changes associated with the binding interactions may be performed while the particles are assembled in a planar array on a substrate, e.g., by taking an assay and a decoding image of the array and comparing the two, e.g., comparing of the assay and the decoding image comprises use of optical microscopy apparatus including an imaging detector and computerized image capture and analysis apparatus. The decoding image may be taken to determine the chemically and/or physically distinguishable characteristic that uniquely identifies the binding agent displayed on the bead surface, e.g., determining the identity of the binding agents on each particle in the array by the distinguishable characteristic. The assay image of the array is taken to detect the optical signature of the binding agent and the analyte complex. In certain embodiments, fluorescent tags (fluorophore dyes) may be attached to the analytes such that when the analytes are bound to the beads, the flourescent intensities change, thus providing changes in the optical signatures of the beads. In certain embodiments, tthe decoding image is taken after the beads are assembled in an array and immobilized and before taking the assay image, preferably before contacting the binding agents on the beads with an analyte. In certain other examples, the binding interactions occur while the beads are in solution, and assembled into an array afterwards and the decoding and assay images are obtained. The identity of the binding agent of the binding agent-analyte complex is carried out by comparing the decoding image with the assay image.

In preferred embodiments, images analysis algorithms that are useful in analyzing the data obtained from the decoding and the assay images. These algorithm may be used to obtain quantitative data for each bead within an array. As summarized in Fig. 7, the analysis software automatically locates bead centers using a bright-field image of the array as a

on apparent serological cross-reactivity between the groups. These groups are termed Cross-Reactive-Groups (CREGs). In current clinical setting, antibodies from a patient are tested against different antigens in individual reactions. Although a reactive pattern of the antibodies can be generated combining the results from different reactions, the competitive nature of interactions between different antibodies and antigens is not reflected in such a pattern. In other cases, several antigens are mixed together for a binding assay. Lack of identification of each antigen in the system prevents generation of a binding profile. The result is only the averaged signal from several antigens. In the bead array system, a panel of different antigens is presented to the antibody analytes in a competitive binding environment, and each antigen can be identified through its association with different types of beads. Thus, binding intensity on each antigen in the competitive reactions can be extracted in a single assay. This co-affinity matrix system will provide binding profiles for the CREGs and greatly advance the understanding of the nature of the reaction and improve the accuracy for the related clinical decisions. For example, a N-antibody and M-antigen system provides a matrix of N x M of possible reactions. It is possible to determine K-nm, the affinity constant governing the interaction between the nth antibody against the mth antigen, where m =1, 2, . . M, and n =1, 2,. . . N. For applications where absolute co-affinity constants are not needed, binding profile will be generated for various antibodies in accordance with the methods of the present invention and results from a patient sample can be matched to these profiles or combination of these profiles.

Co-affinity matrix may also be used to characterize the analyte. For example, combination of the coefficients of the co-affinity matrix and known concentrations of analyte and binding agents participating in the formation of analyte-binding agent complexes serves to define a competitive binding interaction descriptor , e.g.,The molecular interaction parameter,

present invention. In certain embodiments, the particles are assembled using LEAPS, as with non-magnetic encoded beads. The encoded also be used in array generation, and assayed. The present invention also includes the formation of a planar array of encoded and functionalized superparamagnetic particles on a substrate by application of magnetic field to said particles.

Several methods for the synthesis of monodisperse superparamagnetic microspheres are known in the art. G. Helgesen et al., Phys. Rev. Lett. 61, 1736 (1988), for example, disclosed a method which utilizes porous and highly cross-linked polystyrene core particles whose interior surfaces are first nitrated, following which iron oxides are precipitated throughout the particle to produce a paramagnetic core. Following completion of this step, the particles are coated with functional polymers to provide a reactive shell. US patent 5,395,688 to Wang et al. describes a process for producing magnetically responsive fluorescent polymer particles composed of a fluorescent polymer core particle that is evenly coated with a layer of magnetically responsive metal oxide. The method utilizes preformed fluorescent polymeric core particles which are mixed with an emulsion of styrene and magnetic metal oxide in water and polymerized. A two step reactive process such as this suffers from the drawback of possible inhibition of polymerization by the fluorescent dye or conversely bleaching of the fluorescence by the shell polymerization process.

The method also provides a novel process for making color encoded magnetic beads, a simple and flexible one-step process to introduce into preformed polymeric microparticles a well controlled amount of magnetic nanoparticles, prepared in accordance with the procedure described below, along with well controlled quantities of one or more fluorescent dyes. In an embodiment of the present invention, the quantity of the magnetic nanoparticles. is controlled to produce magnetic particles that form an array on a substrate upon application of magnetic field to said particles. This process involves swelling the polymer particles in an organic solvent containing dyes and magnetic nanoparticles and therefore applies to any polymer particle which can be subjected to standard swelling

profiles which were generated and projected onto the substrate by a PC-programmable illumination pattern generator (described in U.S. Serial No. 09/397,793, filed September 17, 1999, which is incorporated herein by reference in its entirety). This drag-and-drop operation reduced the separation between the two sub-arrays from approximately 250 μm to 20 μm. Beads were moved at 5 V_{pp} at a frequency of 2 kHz; total power projected onto the substrate surface was ~5 mW. The combination of chemical and spatial encoding permits a given set of chemical bead markers to be used multiple times and reduces the demands placed on either encoding dimension while facilitating the realization of large coding capacities.

Example 2: Array formation on patterned surface

Illustrated in Fig. 10 is an array of encoded beads assembled on a patterned silicon chip using an AC voltage of 1-2 V_{pp} and a frequency of 100-150 Hz, applied across a 100 μm electrode gap filled with an aqueous bead suspension; a thermal oxide (~1000 Å) on the substrate was patterned by etching the oxide to a thickness of 50-100 Å in a set of square features (~30 x 30 μm^2) on 130 μm centers; arrays of similar layout also can be produced in response to suitable illumination patterns. Each sub-array shown here contains approximately 80 beads coupled with anti-cytokine monoclonal antibodies. Carboxylate-modified polystyrene beads of 5.5μm diameter (Bangs Laboratory, Fishers, IN) were stained with a combination of two types of fluorescent dyes and were then functionalized with anti-cytokine-mAb. The assembly process ensures collection of all beads at the substrate surface. Bead encoding was as follows: IL-2 (Bright Red); IL-4 (Dim Red); IL-6 (Bright Green); M-CSF (Dim Green) and TNF-α (Yellow).

Example 3: Formation of arrays of magnetic particles

Colloidal particles exhibiting a finite diamagnetic susceptibility, when disposed on a planar substrate can be assembled into ordered arrays in response to increasing magnetic fields. Commercially available superparamagnetic particles (Dynal, Oslo, NO), dispersed from a fluid suspension onto the planar surface of the lower of two parallel bounding surfaces of a

substrate. An application of random bead assemblies to determine affinity constants in a multiplexed format is described in Example 6.

Example 5: An automated chip-scale array manufacturing process

As illustrated in Fig. 11, the process involves liquid handling and pipetting of beads onto chips mounted in single-chip cartridges or multi-chip cartridges. Bead arrays are formed using methods such as those in Examples 1, 2 or 3., followed by array immobilization and decoding. The resulting decoding images are stored for later use along with an optional chip ID ("bar code").

Example 6: Determination of affinity constants by post-assay analysis of bead assemblies.

Quantitative binding curves for the cytokines TNF-α and IL-6. Binding curves were generated by performing sandwich immunoassays using chemically encoded beads in suspension, said suspensions being confined to one or more reaction compartments delineated on-chip, or in one or more reaction compartments off chip. By completing the reaction with beads maintained in suspension, assay kinetics similar to homogeneous assays can be attained. Following completion of the binding reaction, beads were assembled on chip to permit multiplexed quantitative image analysis. Random assemblies prepared according to Example 4 or ordered bead arrays prepared according to Example 1 or 2 may be used. An advantage of ordered, dense assemblies produced by the methods of Examples 1 or 2 is the higher spatial density and higher assay throughput attained by processing a greater number of beads.

As an illustration, Fig. 12 displays quantitative binding curves for TNF-α and IL-6, obtained from randomly dispersed beads. A commercial-grade 8-bit video-CCD camera (Cohu, San Diego, CA) was used in a mode permitting multi-frame integration. The range of concentrations of antigen used in the two assays was 700 fM to 50 pM for TNF-α and 2 pM to 50 pM for IL-6. At each concentration, the number of molecules bound per bead was estimated by comparison with calibration beads coated with known quantities of Cy5.5-labeled

Example 7: Genotyping by Polymorphism Analysis

To illustrate the application of the present invention to the implementation of genotyping, Fig. 13 shows the design of the assay in which five pairs of 20-mer binding agents corresponding to four polymorphic regions of a gene were coupled to color-encoded beads. The pairs of binding agents $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$ each display a single nucleotide difference in their respective sequences; the pair $\delta 3$, $\delta 4$ displays a difference of three nucleotides, the binding agents in the set $\gamma 1$, $\gamma 3$, $\gamma 3$, $\gamma 4$ display small insertions and deletions. The ten binding agents were are divided into two subgroups of five which were incorporated into two subarrays. In this example, there are several hundred beads for each type. Following bead immobilization, an on-chip hybridization reaction was performed in TMAC buffer (2.25 M tetramethylammonium chloride, 37 mM Tris pH 8.0, 3 mM EDTA pH 8.0, and 0.15% SDS) at 55°C for 30 min. The analyte is a 254-base PCR fragment produced from a patient sample and fluorescently labeled at the 5'-prime end with BODIPY 630/650 (Molecular Probes, Eugene, OR). Image acquisition was performed after replacing the assay buffer with fresh TMAC buffer.

Fig. 14 shows decoding and assay images for one subarray. Each bead shown in the assay image obtained after hybridization is analyzed to determine fluorescence intensity and bead type; as with the cytokine assay, the latter operation compares assay and decoding images using a template matching algorithm. Fig. 15 displays the resulting intensity histograms for each bead type: in these histogram plots, the horizontal axis refers to relative signal intensity from 0 to 1 and the vertical axises refer to bead numbers. The histograms show that most of the beads displaying probe $\alpha 1$ bind no analyte while most of the beads displaying probe $\alpha 2$ exhibit significant binding; the mean signal level of $\alpha 2$-beads exceeds that of $\alpha 2$-beads by a factor of ~3.2, indicating that analyte contains DNA sequences complementary to $\alpha 2$ but not $\alpha 1$. For the

buffer. After one hour of incubation at 37°C, fresh buffer was added on top of the chip and image acquisition was performed. Antibodies and antigens used in the assays were obtained from R&D Systems (Minneapolis, MN); the secondary antibody was labeled with Cy5.5 using a standard kit (Amersham Pharmacia Biotech, Piscataway, NJ).

The decoding image Fig. 17B shows five types of beads in a false-color display with the same encoding pattern as that of Fig. 10. All beads are of the same size (5.5 μm diameter); the apparent difference in the size of beads of different types in the decoding image is an artifact reflecting different internal bead staining levels and "blooming" during CCD recording of the decoding image. Comparison (using the image analysis methods disclosed herein) of the decoding image with the assay image in Fig. 14A reveals that active beads, of yellow and bright green types, captured TNF-α and IL-6, respectively. This assay protocol has been extended to the following set of twelve cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, TGF-β1, IL-12, EGF, GM-CSF, M-CSF, MCP-1 and TNF-α. The on-chip immunoassay requires no additional washing other than changing reagent solutions between assay steps. Comparison between assay and decoding images shows that two different cytokines were present in the sample, namely IL-6 and TNF-α. The pre-formed arrays described in this example also permit the determination of affinity constants in a manner analogous to the analysis described in Example 6.

Example 11: Aptamers for Protein Profiling

Aptamers may be selected from large combinatorial libraries for their high binding affinities to serum proteins (L. Gold, B. Polisky, O. Uhlenbeck, M. Yarus, *Annu. Rev. Biochem.* **64**: 763-797. (1995)). Random encoded arrays of aptamer-functionalized beads would serve to monitor levels of serum proteins; correlations in binding patterns on the array (see also Example 10) may serve as a phenotype of disease states.

Example 12: Mixed DNA -Protein Arrays

Of significant interest to genomic functional analysis is the fact that the method of the present invention accommodates protein and DNA arrays without change in array

$$\frac{\partial[L_i \cdot R_j]}{\partial t} = k_{on,ij}[L_i]\left([R_{j,0}] - \sum_{n,m}[L_m \cdot R_n]\right) - k_{off,ij}[L_i \cdot R_j] \quad \forall\, i, j,\ L_i \equiv L_i(t,x,0) \tag{2}$$

The first term on the right of Eq. (1) describes the association of ligands and receptors into complexes and involves of concentration of free sites on the surface. The second term describes the disassociation of complexes by way of release of ligands, thereby freeing up receptor sites for further reaction. Since a maximum of (i×j) bimolecular complexes can form, there could be as many boundary conditions generated from the above equation. For the equilibrium case, the left hand-side of Eq. (1) is set to zero, and the matrix of coaffinities, $[K_{ij}] = k_{on,ij}/k_{off,ij}$, can then be defined to accommodate cross-reactivities between multiple species in the bulk and on the surface. In a batch reactor under equilibrium conditions, we may solve the system of differential equations to obtain the number of molecules of each ligand bound on beads of each type.

L_1	Ligand concentration	10 pM
L_2	Ligand concentration	100 pM
R_{01}	Initial receptor concentration	$1x10^4$ /bead
R_{02}	Initial receptor concentration	$1x10^4$ /bead
n_{B1}	Bead number density	$1x10^4$ /ml
n_{B2}	Bead number density	$1x10^4$ /ml
$[K]$	Coaffinity matrix	$[1x10^{11}\ 1x10^9$ $1x10^8\ 1x10^{11}\ ]$ l/mole

As an illustrative example, the ligand distribution has been calculated (from the model in Eq (1)) for a reference set of two ligands and two types of receptors immobilized on

Example 14. Multiplexed Analysis of Reaction Kinetics

As illustrated in the foregoing examples, extensive washing generally is not required to discriminate beads from a background of solution fluorescence. Consequently, assay image sequences may be recorded in a homogeneous assay format to document the evolution of a binding reaction and to determine kinetic data for each of the binding reactions occurring.

Homogeneous binding assays may be performed in simple "sandwich" fluidic cartridges permitting optical microscopic imaging of the bead array and permitting the introduction of an analyte solution into a chamber containing a random encoded array of beads. More generally, the array also may be exposed to an analyte or other reaction mixtures under conditions of controlled injection of fluid aliquots or continuous flow of reactants or buffer. Using theoretical modeling, optimal combinations of relevant performance control parameters of this bead array reactor may be identified to minimize the time to equilibration or to maximize the portion of analyte captured by the array [K. Podual and M. Seul, TM KP-99/02]. Flow rate can be controlled by any of a number of available pumping mechanisms [M. Freemantle, *C&EN*, 77: 27-36].

Table - List of parameters used in simulations (Fig. 18)

Parameter, units	Value
Initial Receptor Coverage $c_{R,0}$, moles/m^2	8×10^{-9}
Vol Flow Rate, Q, μl/s	1.0
Diffusivity, D, cm^2/s	1×10^{-7}
ON-Rate, k_{on}, /(M s)	1×10^{5}
Affinity Constant, K_A, /M	1×10^{11}
"Sandwich" Reactor Gap Size H, mm	0.1
Reactor Length, L, mm	10
Reactor Width, W, mm	10

Methods and apparatus using biochemically functionalized super-paramagnetic particles for sample preparation in molecular and cellular biology and for a variety of enzyme-catalyzed on-bead reactions have been described ["Biomagnetic Techniques in Molecular Biology", Technical Handbook, 3rd Edition, 1998, Dynal, Oslo, NO]. These bead-based methods can be combined with the Random Encoded Array Detection format of the present invention to implement multi-step on-chip assay manipulations.

For example, Fig. 20 illustrates the integration of a sequence of steps in a miniaturized format for multiplexed genotyping using a single chip with multiple compartments. First, cells are captured from a patient sample by affinity selection using functionalized magnetic beads, cells are lysed electrically or chemically in a first compartment, and genomic DNA is captured to the surface of a multiplicity of magnetic beads by non-specific binding; next, beads are collected by magnetic force into a second compartment which is in fluidic contact with the first compartment, within which the beads and DNA are washed with desired buffers; next, beads are further transferred to a location where PCR is performed using bead-coupled DNA as a template; multiple PCR strategies known in the art are available for this step [F. Fellmann, et.al., *Biotechniques,* **21**:766-770]; next, PCR products released into are captured by hybridization to a pre-assembled random encoded array displaying binding agents that are specific to different polymorphisms targeted by the PCR amplification.

The use of encoded magnetic particles in conjunction with the optical programmability of LEAPS confers the ability to form reversibly immobilized arrays and to conduct programmable multi-step assay sequences under conditions in which beads are used in suspension when this is most favorable, for example to enhance reaction kinetics, and arrays are formed in real-time when this is most favorable, for example to provide a highly parallel format for imaging detection and assay read-out.

For example, as illustrated in Fig. 21, the following sequence of steps could be integrated in a miniaturized format for the formation of a cDNA bead array. First, a pool of encoded magnetic beads, each bead type displaying a gene-specific probe, is introduced to an mRNA pool, and mRNA molecules are hybridized to their corresponding beads; next, on-bead reverse transcription (RT) is performed using bead-attached mRNA as template [E. Horenes,

a $CHCl_3$ (Aldrich Chemical Co., Milwaukee, WI) or CH_2Cl_2/CH_3OH mixture (70/30 (v/v)) (Aldrich Chemical Co., Milwaukee, WI). A predetermined amount of polymer beads was washed thoroughly in methanol (3x) and then evaporated dry. Simultaneous incorporation of the fluorescent dye and the iron oxide nanoparticle was achieved by swelling the beads in organic solvent/nanoparticle/dye mixture. The swelling process was completed within ~ 1hr. Following this the polymer beads were separated by centrifugation and washed with methanol(3x) followed by isooctane(2x) and then methanol(2x) and finally redispersed in 0.2% SDS –DI water solution.

2. A method of determining elements of a co-affinity matrix which describes pair-wise analyte-binding interactions in a competitive multiconstituent equilibrium reaction comprising:

providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles,

contacting the binding agents with an analyte molecule so as to allow the analyte to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;

forming a planar array of the particles on a substrate;

detecting the change in the optical signature associated with said particles for each type of the analyte-binding agent complexes formed; and

determining the identity of the binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith, the determining step occurring either before or after the detecting step, and

wherein said change in optical signature for each type of the analyte-binding agent complexes determines affinities characterizing said analyte-binding agent interaction and said affinities providing elements of a co-affinity matrix which describes pairwise analyte-binding agent interactions in a competitive multiconstituent equilibrium reaction.

3. The method of claim 1 or 2, wherein the co-affinity matrix obtained is used to characterize the analyte.

4. The method of claim 3, wherein a combination of the coefficients of the co-affinity matrix and known concentrations of analyte and binding agents participating in the formation of analyte-binding agent complexes serves to define a competitive binding interaction descriptor .

binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;

detecting the change in the optical signature associated with said particles for each type of the analyte-binding agent complexes formed at preset intervals following said preset initial time; and

determining, for each type of analyte-binding agent complex, the association rate constants from the time dependence of said changes in optical signature associated with said particles.

8. A method of analyzing the kinetics of molecular binding interactions governing the dissociation of analyte-binding agent complexes formed between one or more analytes and one or more binding agents, comprising:

providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles, and wherein the particles are arranged in a planar array on a substrate;

determining the identity of said binding agents on each particle in the array by the chemically or physically distinguishable characteristic associated therewith;

contacting the binding agents in the array with an analyte molecule so as to allow the analyte to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a change in an optical signature associated with the particles whose binding agent is involved in the formation of the complex;

detecting the change in the optical signature associated with said particles for each type of the analyte-binding agent complexes formed;

exchanging, at a preset exchange time, the analyte solution with a second solution containing no analyte to allow the bound analytes to unbind from the analyte-binding agent complexes, said unbinding resulting in a change in the optical signature associated with the particles having analyte-binding agent complexes, and

providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles, and wherein the particles are arranged in a planar array on a substrate;

generating a de-coding image of the array showing the location of each binding agent in the array;

contacting the binding agents with a sample that may contain an analyte so as to allow the analyte, if present in the sample, to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a corresponding or a proportional change in the optical signature associated with the particles whose binding agent is involved in the formation of the complex;

generating an assay image of the array which detects the change in the optical signature associated with said particles, and deriving from the change in the optical signature the number of analyte-binding agent complexes formed; and

determining the identity of the analyte in the analyte-binding agent complex by comparing the decoding image with the assay image.

16. A method of performing a bioassay comprising:

providing a plurality of particles comprising at least two different particle populations, each population being distinguishable by a binding agent attached thereto, wherein the particles are associated with a chemically or physically distinguishable characteristic that uniquely identifies the binding agents on said particles,

contacting the binding agents with a sample that may contain an analyte so as to allow the analyte, if present in the sample, to form an analyte-binding agent complex with one or more binding agents, the formation of each complex resulting in a corresponding or proportional change in the optical signature associated with the particles whose binding agent is involved in the formation of the complex;

forming a planar array of the particles on a substrate;

22. The method of claim 21, wherein the array of magnetic particles is assembled by application of a magnetic field to said particles.

23. The method of claim 15 or 16, further comprising calculating the affinity constant of a binding interaction between an analyte and its binding agent from the number of the analyte-binding agent complexes formed.

24. A method of integrating sample preparation and bioassay using magnetic particles comprising:

providing a plurality of magnetic particles comprising at least two different particle populations, each population being distinguishable by a recognition molecule attached thereto, wherein the particles are attached to a chemical characteristic that uniquely identifies a biomolecule of interest that selectively binds to the recognition molecule;

providing a biological fluid containing biomolecules and allowing said biomolecules to interact with the recognition molecules on the magnetic particles;

removing the fluid along with unbound components thereof;

transforming the biomolecules bound to the magnetic particles to produce transformed biomolecules, wherein the transformed biomolecules remain attached to the magnetic particles on which they are synthesized;

performing a bioassay according to the method of claim 15 or 16, wherein the binding agents comprise the transformed biomolecules.

25. The method of claim 24, wherein the biomolecules of interest comprises mRNA and the transforming comprises reverse transcribing said mRNA to produce cDNA, which is attached to the magnetic particles.

26. The method of claim 24, wherein the sample preparation and bioassay occur in the same compartment.

cDNA is released from said magnetic particles after the reverse transcription and transported to the assay compartment and used as an analyte in the bioassay.

31. A method of preparing monodisperse magnetic fluorescent particles comprising providing polymeric microparticles and swelling said microparticles in an organic solvent containing one or more and magnetic nanoparticles to produce magnetic particles, wherein the fluorescent dyes and the magnetic nanoparticles are distributed throughout the magnetic particles without being localized at specific locations with the particle.

32. An array comprising a plurality of magnetic particles comprising at least two different particle populations distinguishable by a chemical characteristic associated therewtih, wherein the magnetic particles are assembled on a planar array in a compositionally random manner.

33. The method of claim 5, wherein the analyte specifically binds to one binding agent but not to other binding agent(s) present.

34. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield [LR], given $[L_o]$, $[R_o]$, and n_B, where $[L_o]$ is the initial analyte concentration, $[R_o]$ is the number of binding agents per bead and n_B is the number of beads.

35. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield [LR], for at least two measurements involving different bead numbers, n_B and given initial analyte concentration $[L_o]$.

36. The method of claim 33, wherein, using the law of mass action, affinity constant K is determined from said change in optical signature following conversion to yield

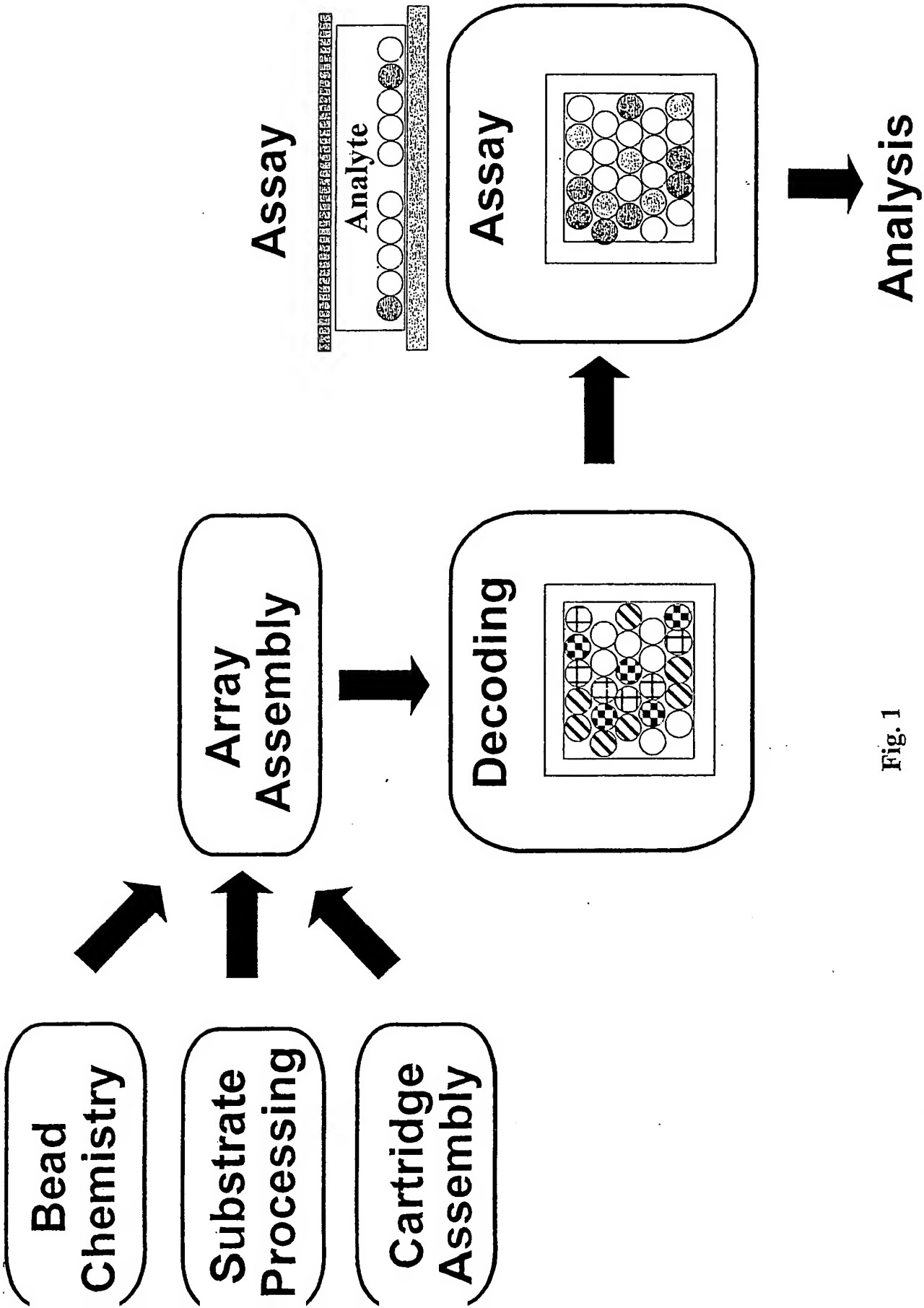

Bead Chemistry
Substrate Processing
Cartridge Assembly
Array Assembly
Decoding
Assay
Analyte
Assay
Analysis

Fig. 1

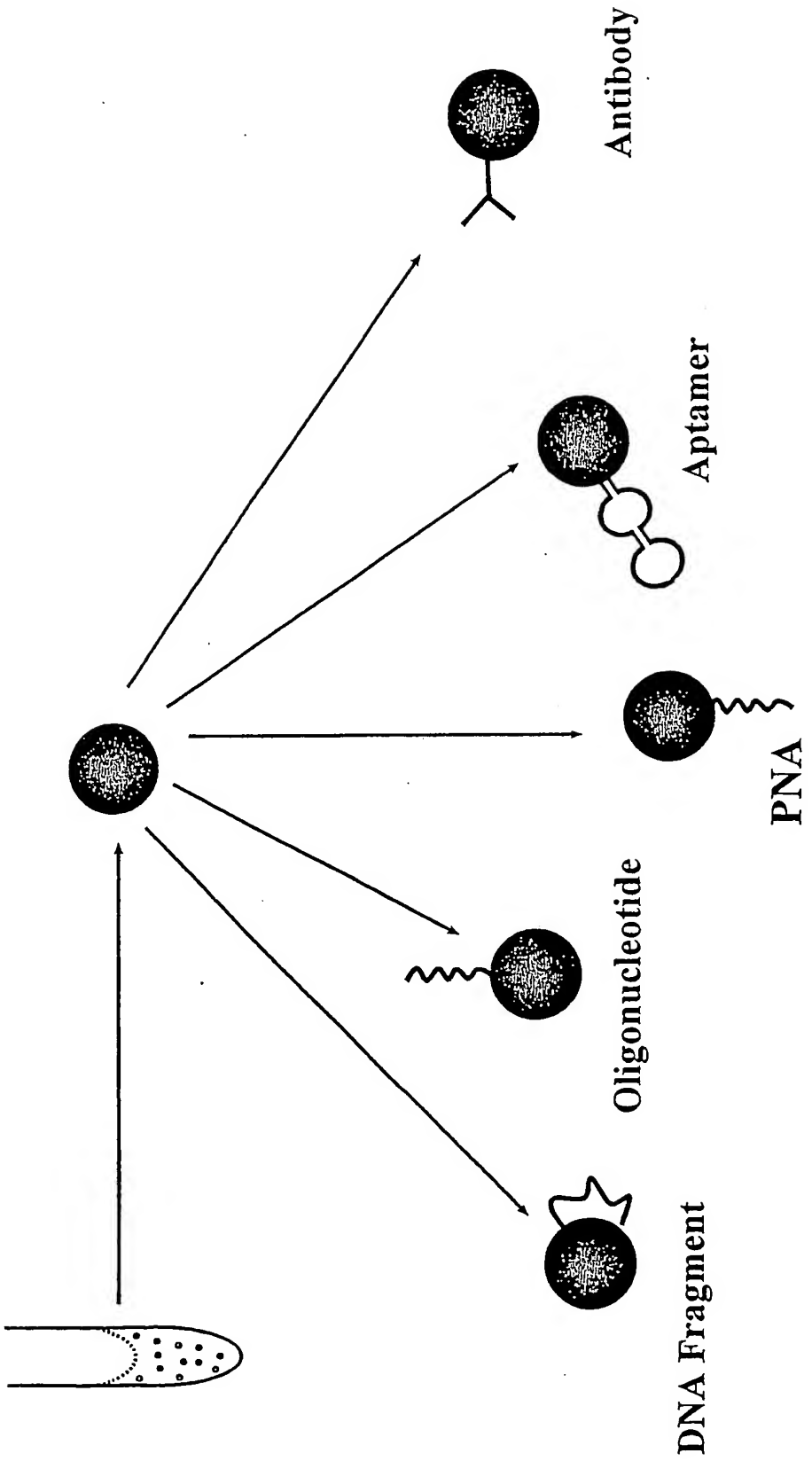


Fig. 2

IN-HOUSE
Wafer
Single chip
Chemically patterned wafer surface
Hydrophobic
Hydrophilic
FOUNDRY
Mask
Process
Etch
Die out
QC
Package

Fig. 3

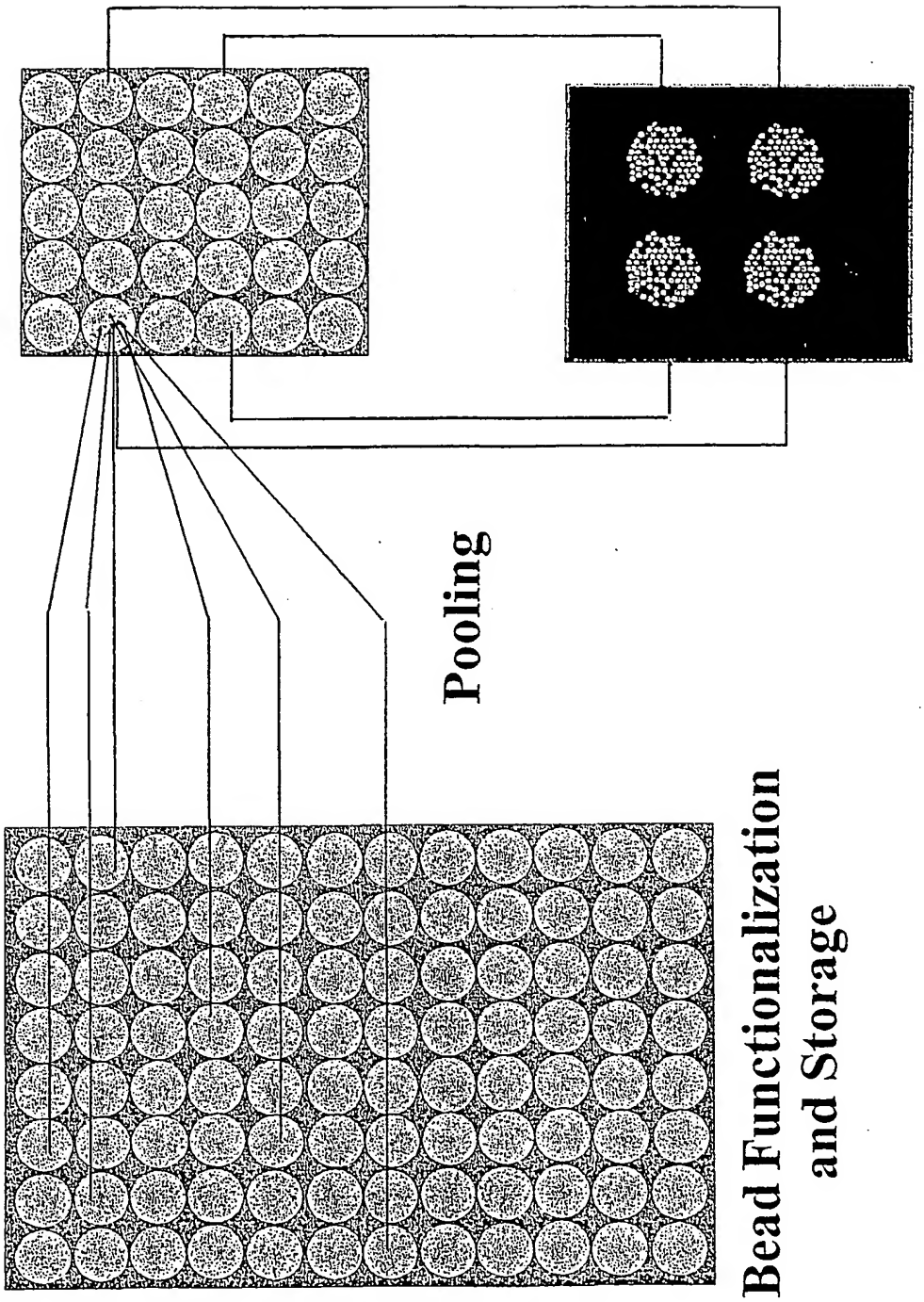

Array Formation
Pooling
Bead Functionalization
and Storage

Fig. 4

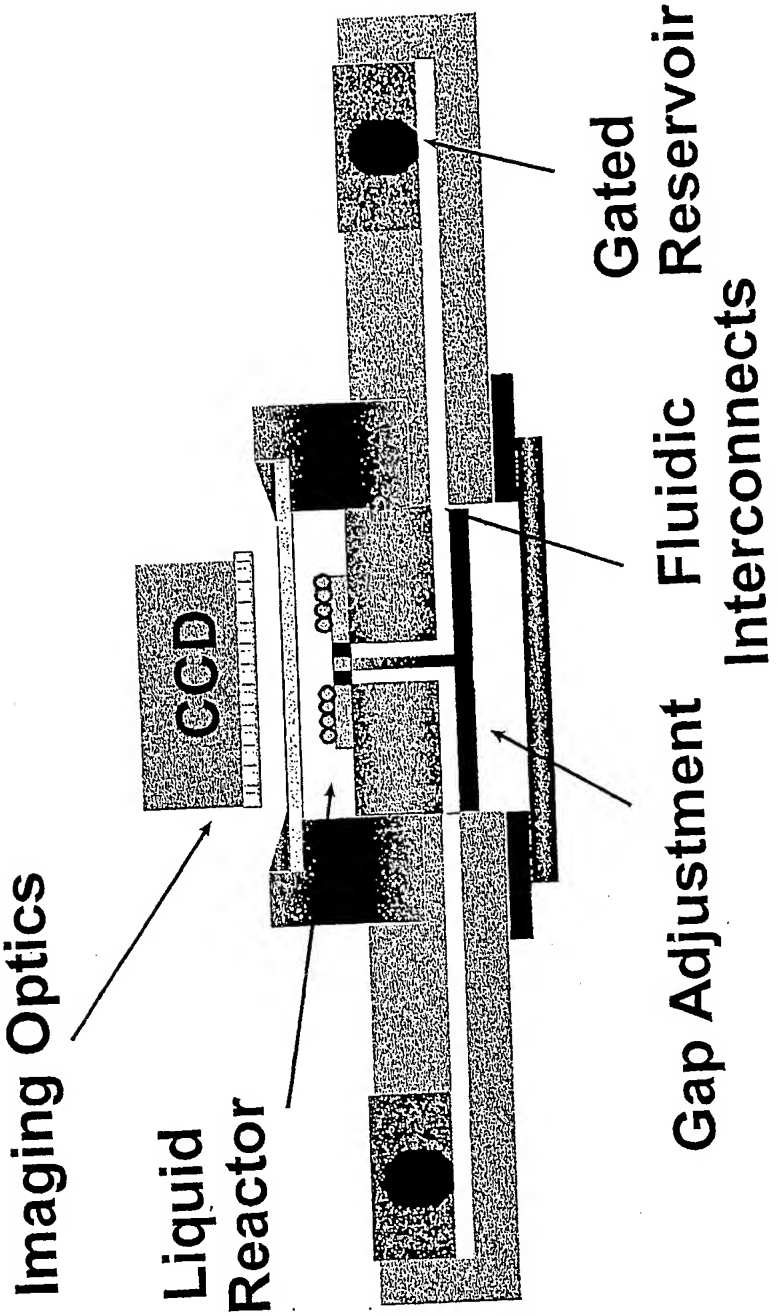

Imaging Optics
CCD
Liquid Reactor
Gap Adjustment
Fluidic Interconnects
Gated Reservoir

Fig. 5

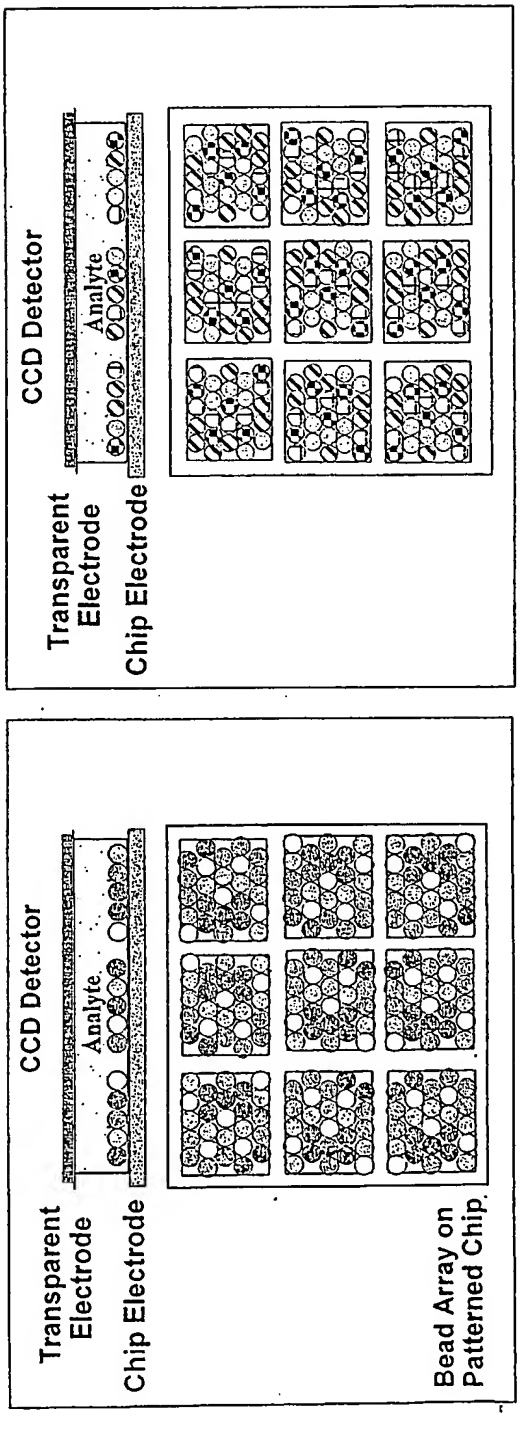

Imaging/Detection
CCD Detector
Transparent Electrode
Analyte
Chip Electrode
Bead Array on Patterned Chip
Decoding of Bead Types
CCD Detector
Transparent Electrode
Analyte
Chip Electrode

Fig. 6

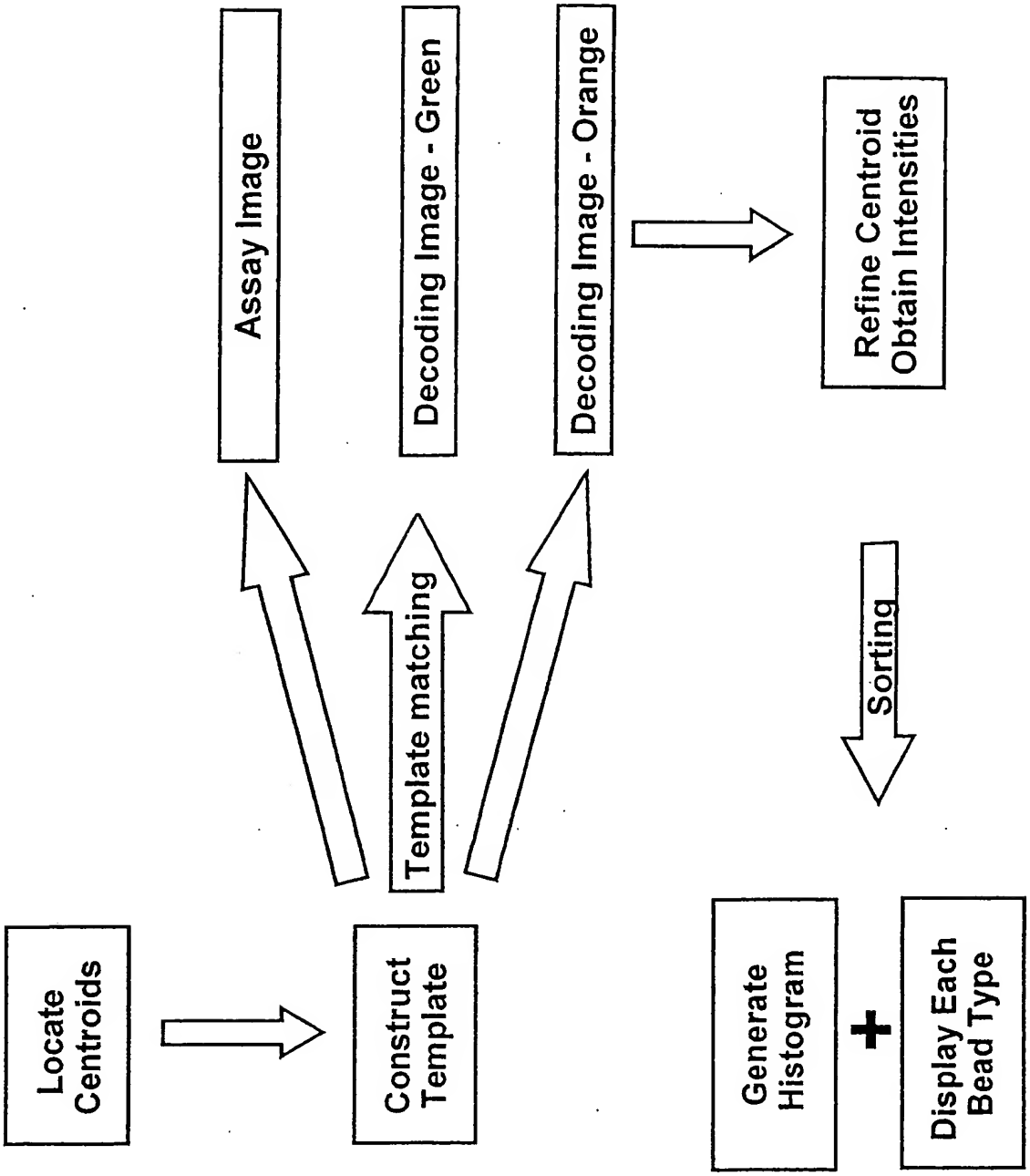

Locate Centroids
Construct Template
Assay Image
Decoding Image - Green
Decoding Image - Orange
Template matching
Refine Centroid Obtain Intensities
Sorting
Generate Histogram
+
Display Each Bead Type

Fig. 7

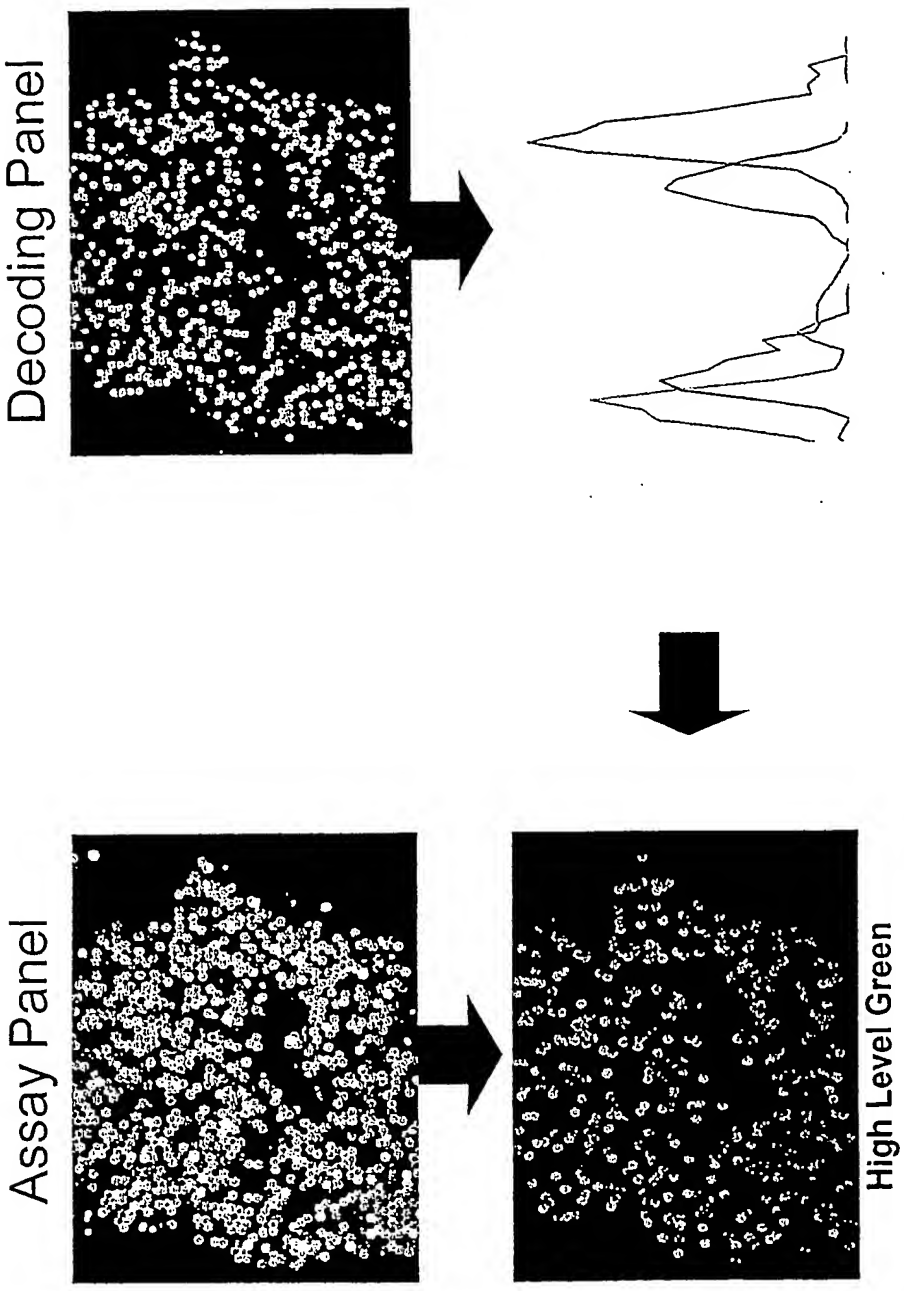


Fig. 8

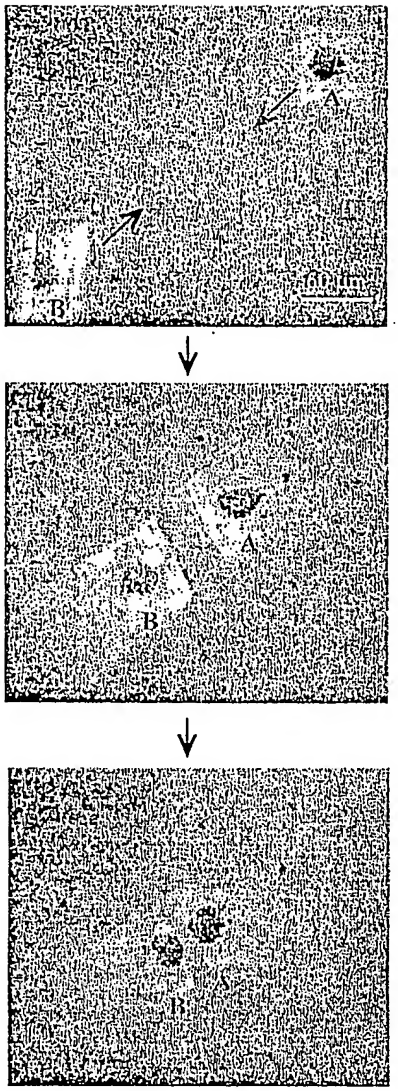


Fig. 9

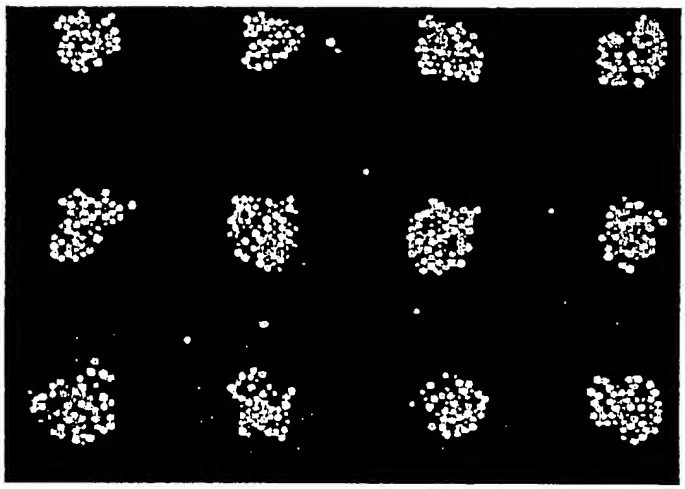


Fig. 10

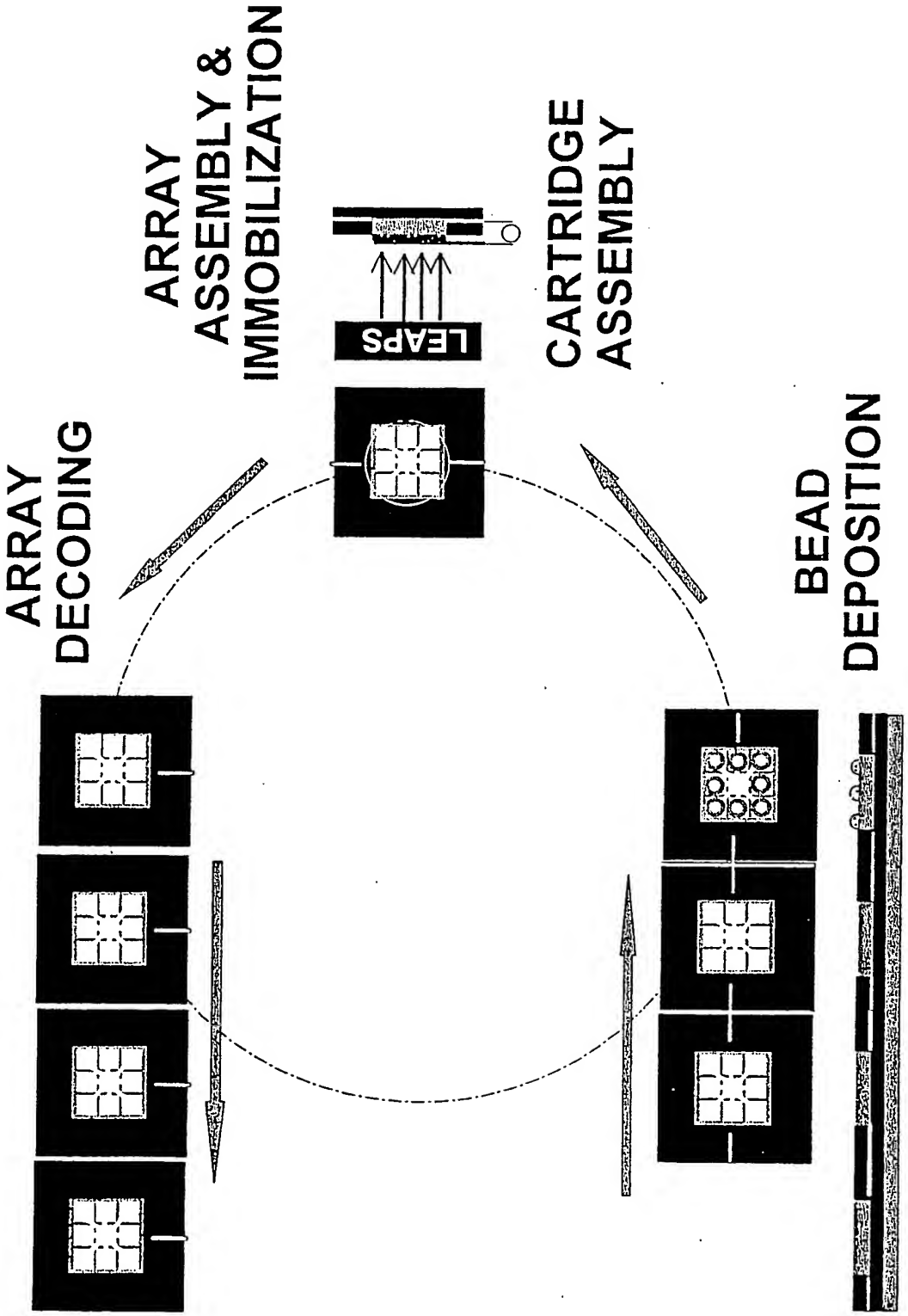

ARRAY ASSEMBLY & IMMOBILIZATION
LEAPS
CARTRIDGE ASSEMBLY
ARRAY DECODING
BEAD DEPOSITION

Fig. 11

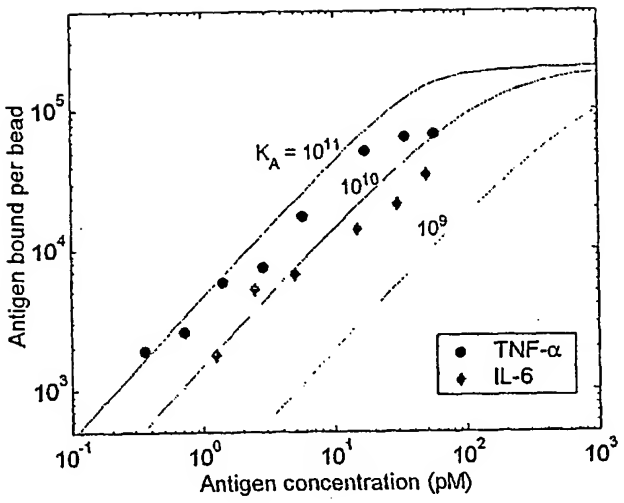

Antigen bound per bead
10^5
10^4
10^3
$K_A = 10^{11}$
10^{10}
10^9
TNF-α
IL-6
10^{-1}
10^0
10^1
10^2
10^3
Antigen concentration (pM)

Fig. 12

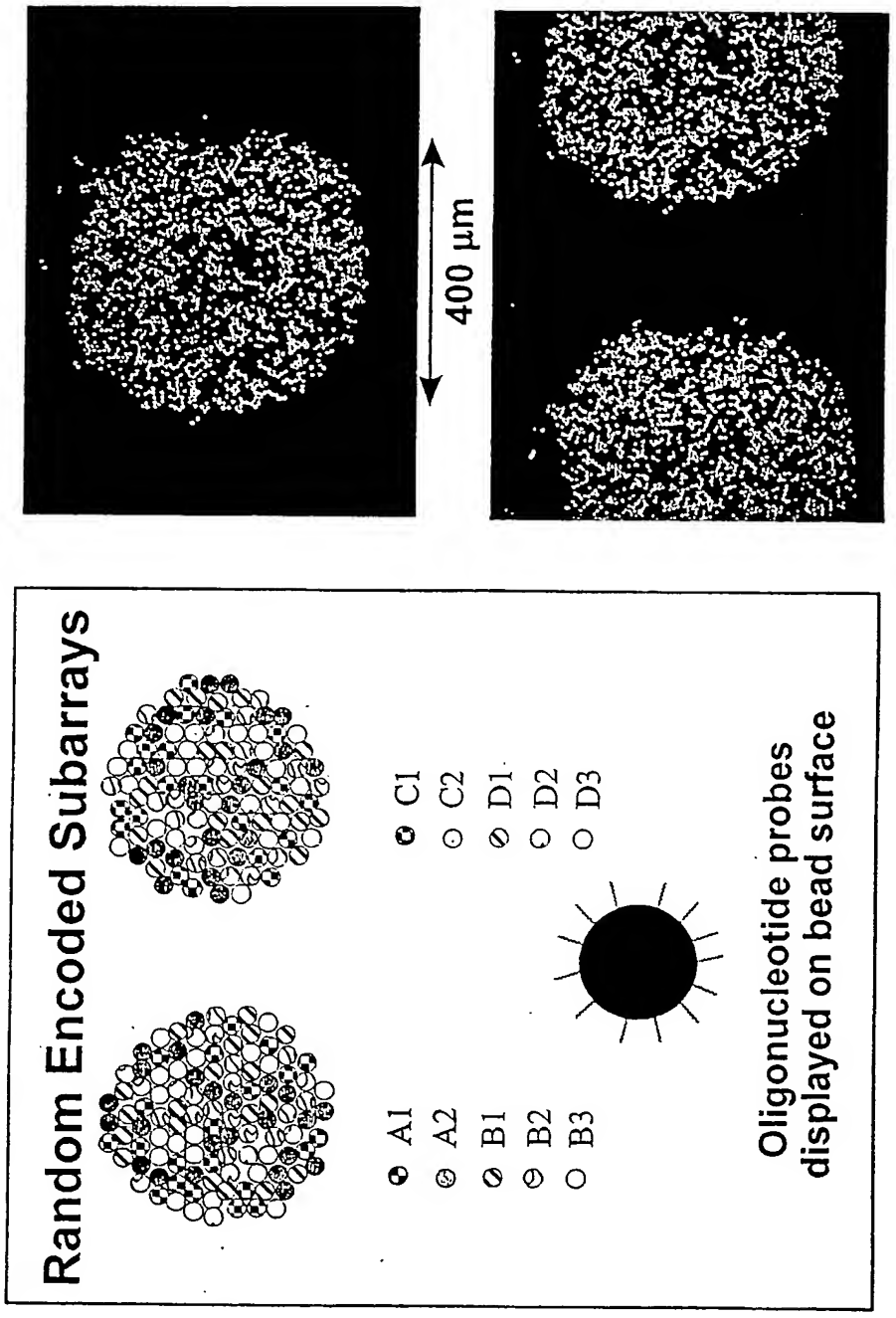

400 µm
Random Encoded Subarrays
A1
A2
B1
B2
B3
C1
C2
D1
D2
D3
Oligonucleotide probes
displayed on bead surface

Fig. 13

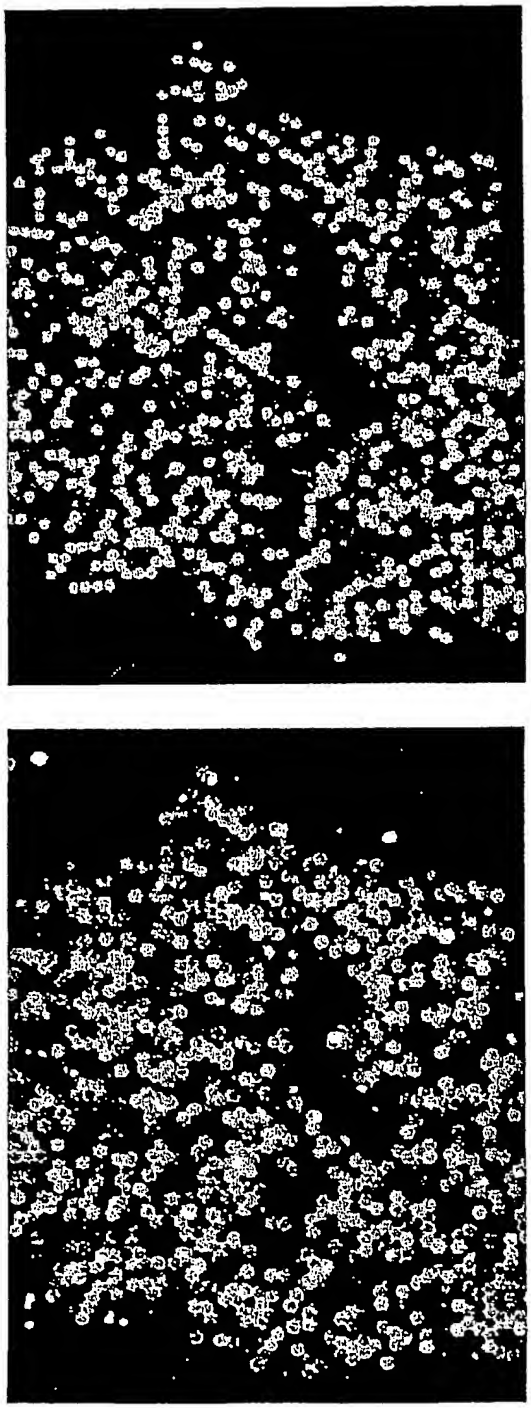


Fig. 14

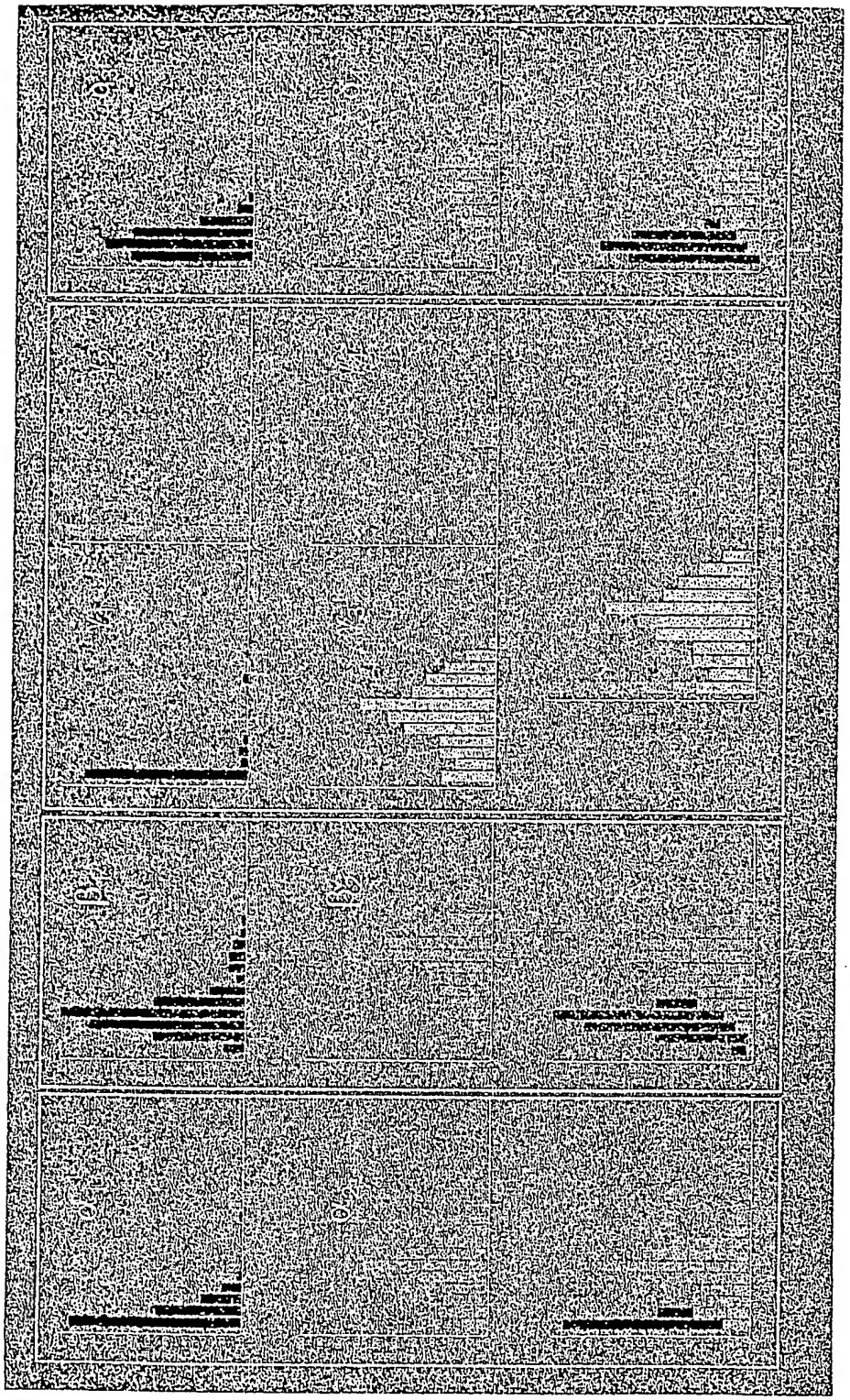


Fig. 15

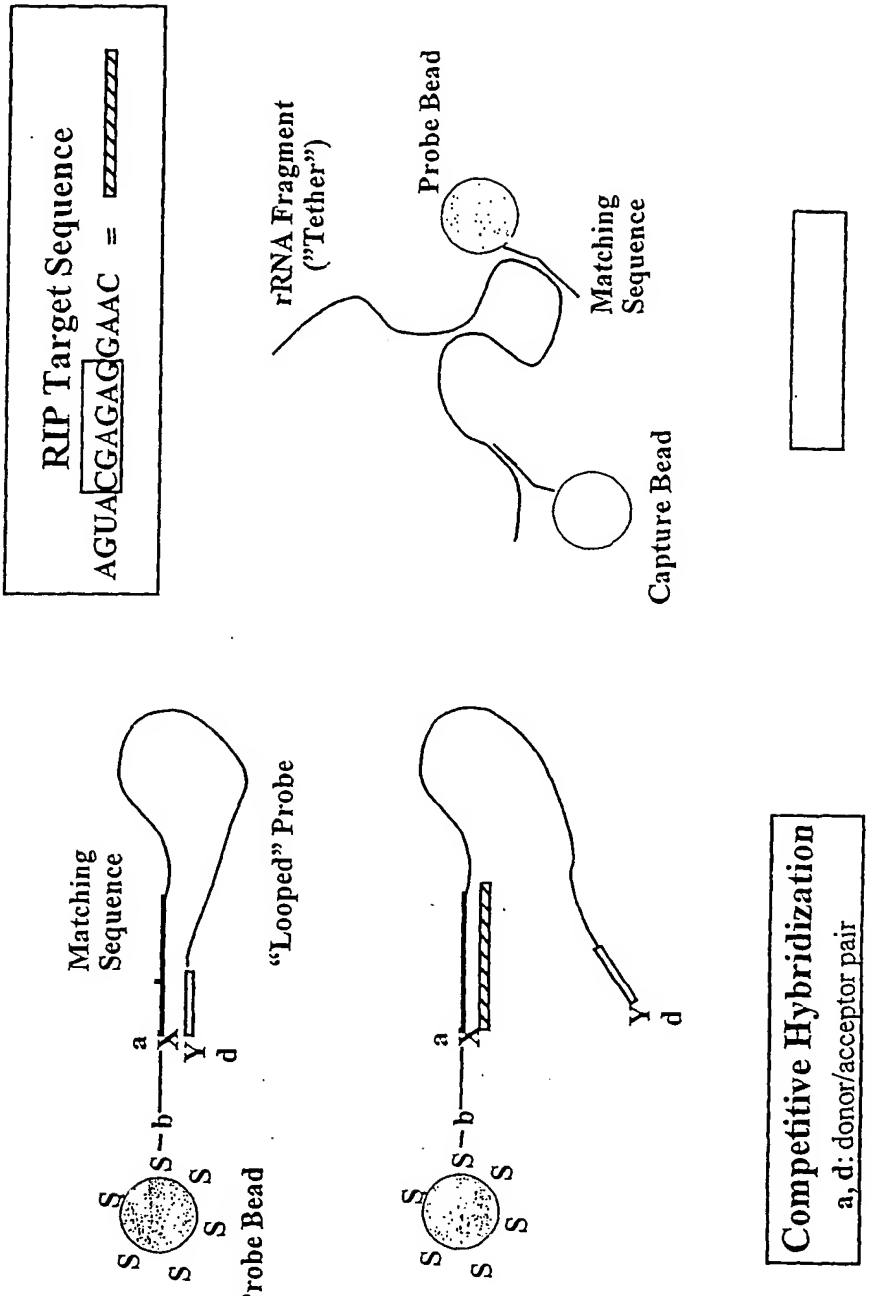

RIP Target Sequence
AGUACGAGAGGAAC =
rRNA Fragment ("Tether")
Probe Bead
Matching Sequence
Capture Bead
Bead-Doublet
Matching Sequence
"Looped" Probe
Probe Bead
Competitive Hybridization
a, d: donor/acceptor pair

Fig. 16

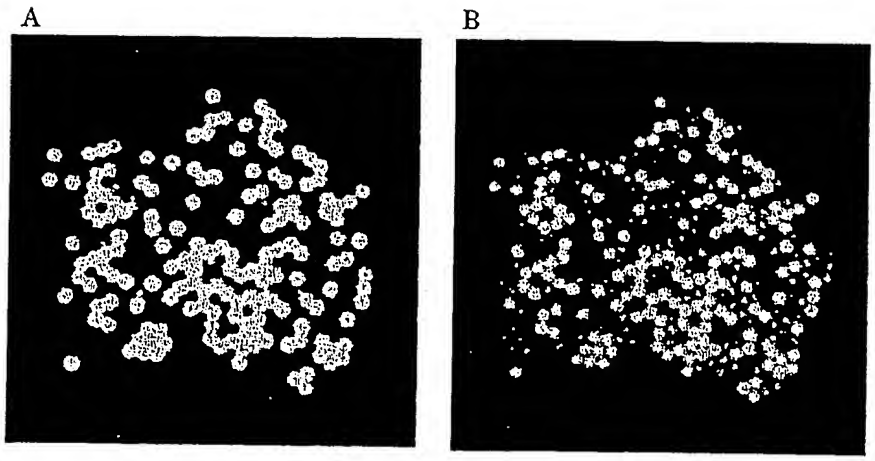


Fig. 17

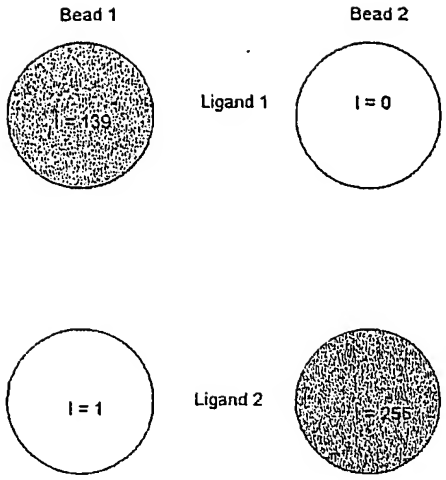


Fig. 18A

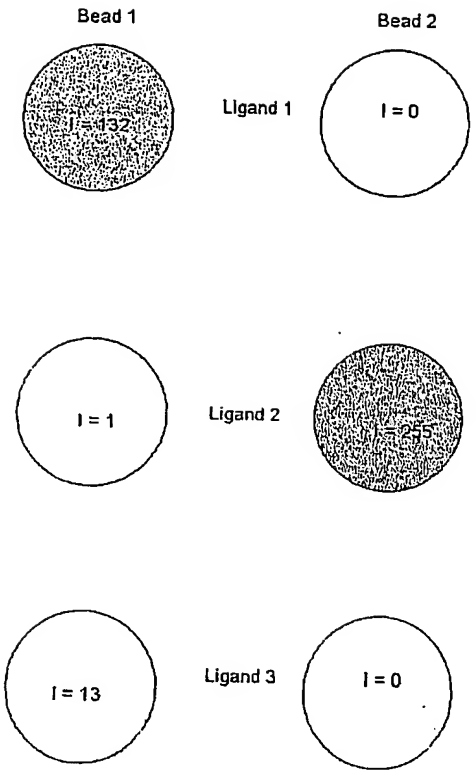
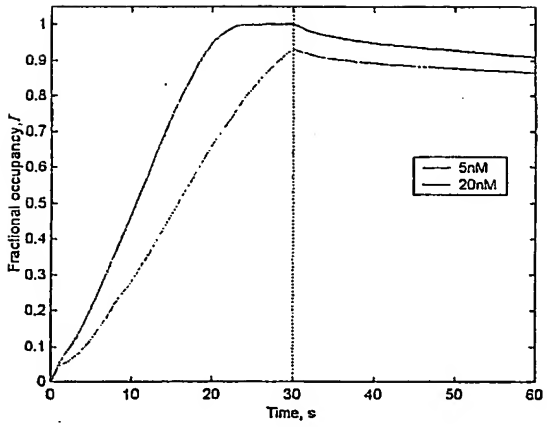


Fig. 18B

(a)


Fractional occupancy, f
Time, s
5nM
20nM
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
10
20
30
40
50
60

(b)

Fig. 19

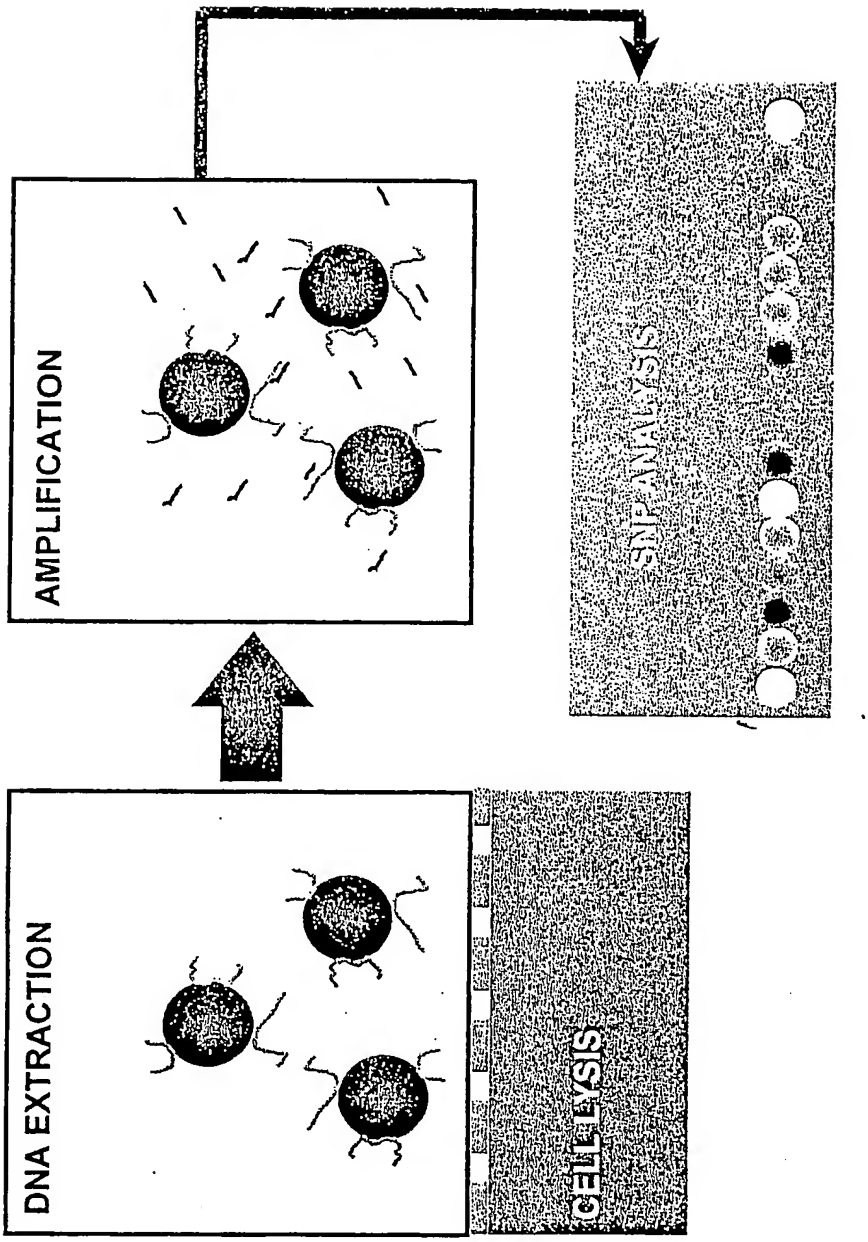


Fig. 20

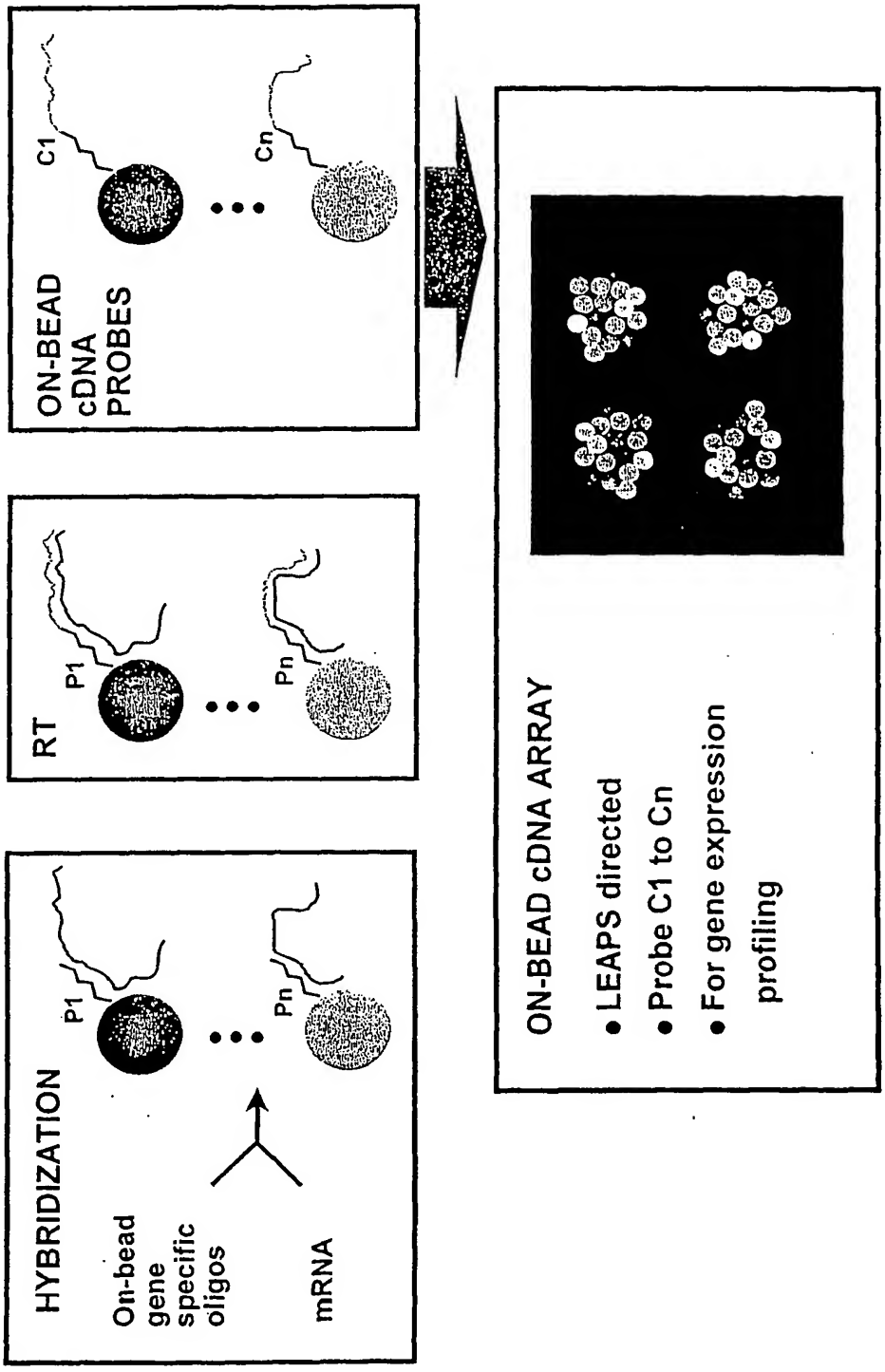

HYBRIDIZATION
On-bead gene specific oligos
mRNA
P1
Pn
RT
P1
Pn
ON-BEAD cDNA PROBES
C1
Cn
ON-BEAD cDNA ARRAY
• LEAPS directed
• Probe C1 to Cn
• For gene expression profiling

Fig. 21